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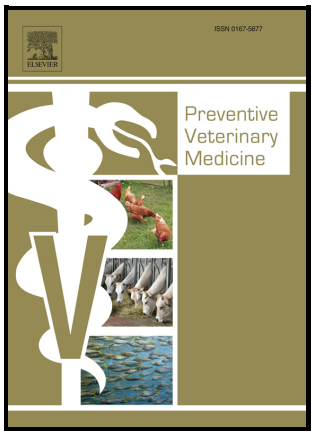

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The seroprevalence of African Horse Sickness virus, and risk factors to exposure, in domestic dogs in Tshwane, South Africa

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Authorship Declaration

The authors confirm that all authors have made substantial contributions to all of the following:

- The conception and design of the study, or acquisition of data, or analysis and interpretation of data.
- The drafting of the article or its critical revision for important intellectual content.
- Final approval of the version to be submitted.

The authors further confirm that:

- The manuscript, including related data, figures and tables, has not been previously published and is not under consideration elsewhere.
- No data have been fabricated or manipulated (including images) to support conclusions.

- This submission does not represent part of a single study that has been split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time (e.g. 'salami-publishing').

The authors confirm that the work submitted is original and does not transgress the plagiarism policy of the journal.

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Author Contribution

All authors JH, ABL, AL AG and GF contributed to the conceptualization, editing and production of this manuscript. JH randomised and selected samples, ABL performed the laboratory testing, JH and GF performed the statistical analysis.

Ethical statement

Ethics approval for this study was granted by the Research Ethics Committee and Animal Ethics Committee of University of Pretoria (REC 043-19). Attached in separate PDF under heading "Title Page (with author details)". The conditions stipulated in the ethical approval were met.

Conflicts of interest

The authors declare no conflict of interest. The submission was blinded and authors unknown to reviewers.

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Abstract

Dogs are the only non-equid species to develop the fatal form of African horse sickness (AHS). Research conducted in 2013 questioned the long-held belief that naturally occurring cases of AHS in dogs were contracted exclusively through the ingestion of contaminated horse meat. *Culicoides* midges, the vector of AHS virus (AHSV) for horses, have an aversion to dog blood meals and dogs were believed to be dead-end or incidental hosts. More recently, dog mortalities have occurred in the absence of horse meat consumption and vector transmission has been suspected. The

current study is a retrospective serological survey of AHSV exposure in dogs from an endemic area. Dog sera collected from dogs (n=366) living in the city of Tshwane, Gauteng Province, South Africa, were randomly selected from a biobank at a veterinary teaching hospital, corresponding to the years 2014-2019. The study used a laboratory in-house indirect recombinant VP7 antigen-based enzyme-linked immunosorbent assay (iELISA) with a test cut-off calculated from AHSV exposure-free dog sera (n=32). Study AHSV seroprevalence was 6% (22/366) with an estimated true prevalence of 4.1% (95% confidence interval (CI) = 1.3% - 8.1%). Incidence was estimated for dogs with multiple serological results with seroconversion occurring at a rate of 2.3 seroconversions per 10 dog years at risk (95% CI = 0.6 - 6.2). A subsection of the study sera was tested with AHSV viral neutralisation test (VN) (n=42) for serotype determination. Antibodies to AHSV serotype 6 were most prevalent (90%) in VN seropositive dogs (n=20) with most dogs seemingly subclinically infected (>95%). Seroprevalence descriptively varied by year and identified risk factors were annual rainfall >754mm (odds ratio (OR) = 5.76; 95% CI = 2.22 – 14.95; $p < 0.001$), medium human population densities, 783-1663 people/km^2 (OR = 7.14; 95 % CI = 1.39 – 36.73; p = 0.019) and 1664-2029 people/km^2 (OR = 6.74; 95% CI = 1.40 – 32.56; p = 0.018), and the month of March (OR = 5.12; 95% CI = 1.41 – 18.61; p = 0.013). All identified risk factors were consistent with midge-borne transmission to dogs. The relatively high seroprevalence and seroconversion rates suggest frequent exposure of dogs to AHSV and indicates the need to investigate the role dogs might play in the overall epidemiology and transmission of AHSV.

Keywords: African horse sickness; serotype 6; *Culicoides imicola*; canine; vector-borne transmission; subclinical infection.

Abbreviations:

AHS: African horse sickness

AHSV: African horse sickness virus

iELISA: Indirect enzyme-linked immunosorbent assay

cELISA: Competitive blocking enzyme-linked immunosorbent assay

BTV: Bluetongue virus

OD: Optical density of the iELISA

S/P: Percentage positivity of test sera to strong positive reference sera of the iELISA

VN: Serum viral neutralisation test for AHSV antibody detection

1 Introduction

African horse sickness (AHS) is a midge-borne, World Organisation for Animal Health (previously known as OIE) listed disease of mostly equids (WOAH, 2022a). African horse sickness is caused by infection with the African horse sickness virus (AHSV), a double-stranded RNA virus of the family *Reoviridae* and genus *Orbivirus* (WOAH, 2022a). The AHSV has nine serotypes and can cause up to 95% mortality in susceptible horses (Dennis et al., 2019). African horse sickness has a significant economic cost estimated at US$95M per annum, due to mortality, disease prevention and control, and barriers to trade (Redmond et al., 2022). The disease is endemic to

sub-Saharan Africa and occasional outbreaks have occurred outside of Africa, including in Thailand and Malaysia in 2020 and predicted to spread into southern China (Dennis et al., 2019; King et al., 2020; Bunpapong et al., 2021; Gao et al., 2022; WOAH, 2022b). The primary vector of AHS is the *Culicoides imicola* midge, which is distributed across most of sub-Saharan Africa as well as southern Europe, the Middle East, and South-East Asia (Leta et al., 2019). The vector distribution appears to be expanding due to global warming and favourable climatic conditions (MacLachlan and Guthrie, 2010; Leta et al., 2019).

Clinical cases in horses are confirmed through the detection of AHSV antigen, or more commonly, genomic RNA detection using reverse transcriptase PCR (Guthrie et al., 2013; Dennis et al., 2019; WOAH, 2022c). AHSV-specific antibodies in equines can be detected using indirect ELISA (iELISA), competitive blocking ELISA (cELISA) and viral neutralisation tests (VN) (WOAH, 2022c). The gold standard AHS serological assay and traditionally favoured method of antibody detection is VN (Maree and Paweska, 2005). Viral neutralization testing is serotype-specific (individual testing for each of the nine serotypes), labour intensive, requires access to reference viruses, and is only available in specialised laboratories. These factors and the relatively high cost restrict the use of VN in serological surveys. The cELISA and iELISA are not serotype-specific and are better suited for serological surveys because of their potential for high throughput, and high sensitivity and specificity in horses and substantially lower cost (Maree and Paweska, 2005; Dennis et al., 2019; WOAH, 2022c). Viral neutralization, cELISA and iELISA have all been previously used in dogs for AHSV antibody detection (McIntosh, 1955; Alexander et

al., 1995; van Sittert et al., 2013; Whitehead et al., 2018) despite not having been validated for use in dogs.

Dogs (*Canis lupus familaris*) are the only non-equid species that have been reported to develop the fatal pulmonary form of AHS (Theiler, 1906; Piercy, 1951; van Rensburg et al., 1981; van Sittert et al., 2013; O'Dell et al., 2018). Outbreaks of AHS in dogs have been reported intermittently and AHS is almost invariably fatal for the dog when clinical signs have developed (Bayley, 1856; Bevan, 1911; Piercy, 1951; Haig, 1956; van Rensburg et al., 1981; van Sittert et al., 2013; O'Dell et al., 2018; Whitehead et al., 2018). Until recently, all known naturally occurring AHS cases in dogs were attributed to dogs consuming AHSV-infected horse meat (Bevan, 1911; Piercy, 1951; Haig, 1956; van Rensburg et al., 1981). However, studies conducted by van Sittert et al. (2013), O'Dell et al. (2018) and Whitehead et al. (2018) all reported AHS cases in dogs in the absence of horse meat consumption raising the possibility of a vector-borne AHSV transmission to dogs (van Sittert et al., 2013; O'Dell et al., 2018; Whitehead et al., 2018).

Due to the low preference of vector midges for canine blood meals, dogs were considered an incidental or dead-end host of AHSV (Mellor and Boorman, 1995; Braverman and Chizov-Ginzburg, 1996; Dennis et al., 2019). However recent studies suggest that midges are less selective feeders than previously thought, and feed on any available hosts in absence of the preferential host species (Hopken et al., 2017). Dog blood has been found in blood-fed midges, including the primary vector C. imicola (Slama et al., 2015; Martínez-de la Puente et al., 2017; Riddin et al., 2019).

Midge-borne transmission of Bluetongue, a closely related *Orbivirus*, has occurred with 21% of dogs testing seropositive for the virus (Oura and El Harrak, 2011). There is clear evidence that midges will occasionally feed on dogs but factors influencing this choice of host are unknown. It is still unknown whether the dog can act as a natural host for AHSV and develop viraemic levels sufficient for the onward transmission to vectors (Oura, 2018).

The increasing incidence of canine AHS mortality in an endemic (and current study) area (O'Dell, 2017) raised concerns that AHS could be an emergent canine disease including a possible canine-adapted AHSV serotype/variant (O'Dell et al., 2018). Recent dog mortalities without access to horse meat (van Sittert et al., 2013; O'Dell et al., 2018; Whitehead et al., 2018) and evidence that midges feed on dogs challenge the assumption that dogs play only a limited role in AHS epidemiology and this should be re-examined (Oura, 2018). It has been suggested that dogs no longer be considered dead-end hosts of AHSV but rather considered hosts with the potential of onward transmission (Oura, 2018).

Dogs imported from endemic areas are not subject to AHS control measures imposed on equids (WOAH, 2022a) and are more frequently exported than horses from endemic countries (European, 2021). No risk assessments could be identified in the literature assessing the risk of AHSV introduction from importing domestic dogs from endemic areas. Assessing the significance of this risk requires knowledge of the number of dogs subclinically infected in endemic areas, the level and duration of viraemia in AHSV exposed dogs and factors influencing competent vectors feeding

on dogs (Oura, 2018). The most recent population-based AHSV seroprevalence study in dogs was reported in 1995 (Alexander et al., 1995) and more current data are required.

The primary aim of the current study was to estimate dog AHSV seroprevalence and identify the prevailing serotypes in an endemic area. An additional objective was to identify risk factors associated with AHSV seropositivity in dogs.

2 Materials and methods

2.1 Ethical statement

Ethics approval for this study was granted by the Research Ethics Committee and Animal Ethics Committee of University of Pretoria (REC 043-19). Written permission to use samples and records collected during routine investigation for research purposes is obtained upon registration of patients at Onderstepoort Veterinary Academic Hospital (OVAH). For samples collected in Cape Town, dog owners informed written consent was obtained for each individual dog sampled.

2.2 Study area

This study was a retrospective, cross-sectional serological survey of AHSV exposure in dogs living in the city of Tshwane, Gauteng Province, South Africa. The area is endemic for AHS with equine cases occurring yearly, and has a population of 2.92 million people, in an area of 6298km^2 and an average population density of 460

people/km^2 (Lehohla, 2015). Tshwane has a humid subtropical climate with mild winters, summer rainfall, and an annual rainfall of 674mm (SAWS, 2021). Tshwane is divided into seven regions and 105 electoral ward districts with approximately 30000 people living in each ward. Low ward population density is found in rural plots or farmlands areas, where livestock, horses and wildlife are kept. Medium population density is prevalent in suburban areas with separate houses and gardens. High population density areas are characteristically apartment blocks or township areas. The number of horses in Gauteng Province is 32714 (Government, 2017) and between 7000 - 11000 are estimated to live in the city of Tshwane (25% and 34% of the population and landmass). Using a dog-to-person ratio of between 1:12 and 1:16 (McCrindle et al., 1997; Conan et al., 2017), it can be estimated that 182000 – 240000 dogs live in the City of Tshwane.

The City of Cape Town falls within the AHS free zone and AHS surveillance zone, of the AHS control area of the Western Cape Province, South Africa (Supplementary Figure 1). Dogs living in this area were considered AHSV exposure-free for the determination of an appropriate iELISA cut-off in dogs.

2.3 Sample size and sample selection

Sample sizes were calculated using an open-source statistical software program (Epitools, Ausvet, Sergeant, ESG, 2018, found at http://epitools.ausvet.com.au). The required sample size was 384 for estimation of population seroprevalence. As no prior expected seroprevalence was known, the expected frequency of 50% was selected for a maximal number with an acceptable margin of error of 5%. Sample sera were selected from a biobank of frozen sera (-80°C) kept at the Clinical

Pathology Section of the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria, Onderstepoort, South Africa, The biobank stores leftover sera from diagnostic tests collected from privately owned dogs during regular veterinary work at OVAH. Study sera were randomly selected from the biobank for the period November 2014 (oldest available samples) to April 2019. Inclusion criteria were dogs greater than a year old from the city of Tshwane. Sera were excluded if there was low volume (<0.5ml) or if it was poor quality (cloudy, blood-contaminated or poorly labelled) on visual inspection. Random selection of multiple specimens taken from the same dog was permitted with test results used for determining the seroconversion rate. For prevalence calculations, the test result from the earliest sera collection date was used with one sample per dog contributing to these estimates.

The number of dogs to test from Cape Town within the AHS-free and AHS - surveillance zone, was based on a freedom of disease calculation with a set design prevalence of 10% and the desire to detect seropositivity at the 95% level of confidence. These sera were used for AHSV iELISA cut-off determination. Sera (n=32) were prospectively collected from privately owned dogs, over a year old, between July and August of 2020, at three participating practices in the City of Cape Town. Dogs that had ever travelled outside the City of Cape Town were excluded.

The number of dogs to test for VN serotype detection was also based on a freedom of disease calculation with a set design prevalence of 10% and the desire to detect specific serotype(s) at the 95% level of confidence. For serotype detection, sera with the highest rank order iELISA S/P values (n=33) were selected for VN testing.

Additionally, VN tests were performed on sera with low iELISA S/P values (n=9) randomly selected from the 50th -150th rank order.

The AHS serological assays were performed according to standard test protocols at the WOAH-approved AHS reference laboratory at the Virology Section of the Onderstepoort Veterinary Institute, Agricultural Research Council, Onderstepoort, South Africa (Maree and Paweska, 2005; WOAH, 2022c). All sera tested were separated from blood clots, stored frozen (less than -20°C) in serum tubes or plain vacutainers and transported in sealed containers on ice packs.

2.4 Indirect enzyme-linked immunosorbent assay (iELISA)

A previously described indirect ELISA was performed with slight modifications (Maree and Paweska, 2005). In brief, positive and negative controls, and test sera, were diluted 1/100 and loaded onto AHSV recombinant VP7 antigen coated ELISA plate wells and incubated for 1 hour (±5 minutes) at 35-39 °C. Diluted protein G - horseradish peroxidase (HRP) conjugate (Invitrogen, USA) was then added to each well, and the plates were similarly incubated. TMB (3,3',5,5'-Tetramethylbenzidine) ready-to-use substrate (Life technologies -Thermo Fisher Scientific, USA) was subsequently dispensed into the wells, followed by incubation at room temperature (18-25 ºC) for 10 minutes (±2 min). The reactions were stopped by adding sulphuric acid (H_2SO_4) solution. All reagents were used at 100µl volumes and evenly dispersed by gentle tapping of the plates. The first two steps were each followed by thorough washing with wash buffer and aspiration of all liquid contents. The absorbance of the samples and controls was measured at 450 nm using an ELISA microplate reader

(BIOTEK ELx 808, Software system, Gen 5.1). Results were presented as percentage positivity to strong positive reference sera, S/P value, where *[S/P =100 x (Sample OD - negative control OD) / (strong positive OD - negative control OD)]*

2.5 Viral neutralization (VN)

Sera were diluted in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, South Africa), from 1:5 to 1:640. Each sera dilution (50ul) was dispensed in duplicate wells in 96-well microtitre plates, followed by the addition of equal volumes of AHSV at a titre of 200 - 300 $TCID_{50}$/ml. The sera and virus mixtures were incubated for one hour at 37^0C and 5% CO_2, following which Vero cells (100ul) at approximately 1X 10^4 cells/ml were added to each well, and the plates incubated as before. Cell and virus control plates were prepared as well. The plates were examined daily under a light microscope for the development of cytopathic effect (CPE). Test wells with no, or, CPE below 25% were considered positive, and the titre of a sample was expressed as the reciprocal of the highest dilution at which no, or less than 25% CPE was observed. The procedure was performed for all nine AHSV serotypes.

2.6 Secondary data collection

Dog age, breed, sex, sterilization status, residential area, and diagnosis/ reason for the visit of the dogs were extracted from electronic hospital records. Monthly rainfall data were obtained from the South African Weather Service. Human population data for Tshwane was based on Statistics South Africa Census 2011 (Lehohla, 2015) and

ward map delineations were obtained from the City of Tshwane (www.tshwane.gov.za Map and GIS section). Reported AHS equine cases were sourced from the Department of Agriculture, Land Reform and Rural Development, South Africa, (found at www.dalrrd.gov.za Animal Health disease reporting).

2.7 Statistical analysis

The iELISA seroprevalence was calculated using the results of the first sera sample collected in situations when dogs were tested more than once. The employed S/P cut-off for the iELISA was determined using the Cape Town sera (AHS-free location) calculated as [$Cutoff = \bar{x} + 2SD$] ($\bar{x}$: mean of the negative control sera S/P values, SD: the standard deviation of the negative control sera S/P values). Based on this cut-off value, the iELISA specificity, Sp_{ct}, was estimated relative to Cape Town sera, and test sensitivity Sn_{vn} was estimated relative to VN as the gold standard. Estimated true prevalence (π) was calculated using the Rogan Gladden estimation as [$\pi = (\hat{p} + (1-Sp_{vn})) / (Sn_{vn} + (1-Sp_{vn}))$]. Results were reported with Blaker's 95% confidence interval (CI) (Reiczigel et al., 2010) calculated using an open-source calculator (Epitools). Where dogs were tested more than once, seroconversion rate (*IR*) was calculated. [$IR = n_1 /((t_1/2)+t_2)$] (t_1: time observed in years in seroconverting, t_2: time observed in dogs seronegative tested more than once).

Continuous variables were categorised in quartiles, apart from rainfall measures that were trichotomized evenly for evaluation of risk factors. Univariate logistic regression was used to screen potential risk factors for associations with dog seropositivity. Where a linear trend was observed on categorical classification, the

variable was evaluated as a continuous variable. Variables containing categories with p-values of <0.25 were dichotomised according to that level and retested. Correlations between continuous variables were examined and Spearman's rho values >0.7 in absolute value were considered collinear and variables of most biological interest were retained for multivariable analysis. Multivariable analysis was performed starting with all variables with p-values <0.25 in univariate analysis. Multiple logistic regression was performed step-wise and variables with the highest Wald p-value were removed one by one until all variables had p-values below 0.05 (backward stepwise method). Fit of multivariable models was evaluated using Hosmer and Lemeshow tests.

Agreement between the iELISA and VN classification was estimated using Cohen's kappa statistic (*K*). The degree of correlation was defined as slight, fair, moderate, substantial, and almost perfect based on *K* values of <0.2, between 0.21 and 0.40, between 0.41 and 0.60, between 0.61 to 0.80 or ≥0.81 respectively (Landis and Koch, 1977). Receiver-operating characteristics (ROC) analysis was performed for the iELISA relative to VNT as the reference standard.

All statistical tests were performed using the statistical program SPSS (SPSS, Version 28, IBM) unless stated otherwise. Statistical significance was defined as p <0.05. For sample size, calculation and population estimate of seroprevalence Blaker CI the open-source calculator was used in Epitools (Sergeant, ESG, 2018. Epitools Ausvet. Found at: http://epitools.ausvet.com.au)

3 Results

3.1 Descriptive results

The 384 study sera originated from 366 individual dogs, with 17 dogs (35 sera) tested more than once. The mean age was 7.1 years (SD 3.9) and the mean weight was 20.7kg (SD 15.8). The male: female ratio was 0.76 with female intact, female spayed, male intact, male castrated and not recorded accounting for 14%, 41%, 20%, 23% and 2% of dogs tested respectively. The most common breeds were Jack Russel terriers (n=31), Labrador retrievers (n=28), Dachshunds (n=26), Yorkshire terriers (n=18) and Boerboels (n=17). The majority (69%) of dogs tested lived within a 10km radius of the study hospital.

3.2 Prevalence and incidence

The iELISA test AHSV seroprevalence was 6.0 % (n = 22/366; 95% CI =4.0% - 8.9%) with an estimated true prevalence of 4.1% (95% CI = 1.3% - 8.1%). Of dogs tested more than once, 18% (n = 3/17) seroconverted (none were seropositive at the first sampling) with a rate of 2.3 seroconversions per 10 dog years (95% CI = 0.6 - 6.2). Two distinct periods of seroconversion were descriptively identified, February 2014 to February 2016, and June 2016 to April 2017 (Supplemental Table 1).

3.3 Serotype detection

Twenty dogs (20/42) were VN seropositive (titre >1:5). Serotype 6 was detected in 90% (n=18) of VN seropositive dogs. The overall median titres were 1:10 and ranged

from 1:5 to 1:40 (Table 1). The majority of VN seropositive dogs were sampled in 2017 (60%). Four sera samples were positive for multiple serotypes of 1,2,3,4,6; 1,7,9; 3,4 and 6,9. All seropositive samples came from the highest S/P value (S/P >0.250 Range: 0.399 to 32.18) selection and none from the lower S/P values (S/P <0.250 Range: -0.398 to 0.15) (Figure 1).

3.4 Description of seropositive dogs

The locations where seropositive dogs lived were widely distributed. There was no apparent geographic epicentre for seropositive cases when accounting for the number of sera tested per ward (Figure 2). Higher seroprevalence occurred in medium ward densities with very few seropositive dogs from wards with low or high population densities (Figure 3). Dog sera from 2014, 2015, 2016, 2017, 2018 and 2019 were seropositive in 0% (0/9), 0% (0/74), 5.1% (4/79), 14.3% (13/91), 4.9% (4/81) and 3.1% (1/32) of dogs tested respectively. The fluctuations in seroprevalence appeared linked to annual rainfall data (Figure 4; Supplementary Figure 2). Sera collected in March and April recorded higher seroprevalences than in other months, following a similar trend to AHS reporting in horses (Figure 5).

3.5 Risk factors for AHSV seropositivity

Significant associations were identified (Table 2) between seropositivity and blood collection in 2017 (OR 4.93, 95% CI: 2.03, 11.95, p <0.001), annual rainfall as a continuous variable (OR = 1.00; 95% CI =1.00 – 1.01; p = 0.007) and above 754mm (OR = 4.27; 95% CI = 1.73 - 10.33; p = 0.001). The year of blood collection (2017) and annual rainfall (>754mm) were collinear (Spearman's rho = 0.923) and annual

rainfall was selected for multivariable analysis. No univariate associations were detected for breed, age, weight, sex, sterilisation status, reason for visit, season, monthly rainfall or seasonal rainfall (Supplementary Table 2). Multivariable modelling identified associations with high annual rainfall (OR = 5.76; 95 % CI = 2.22 – 14.95; $p < 0.001$), middle two human population densities quartiles (OR = 7.14; 95 % CI = 1.39 – 36.73; p = 0.019 and OR = 6.74; 95% CI = 1.40 – 32.56; p = 0.018), and sera collected in March (OR = 5.12; 95% CI = 1.41 – 18.61; p = 0.013). The Hosmer and Lemeshow test ($\chi^2 = 2.054$; degrees of freedom (df) = 6; p = 0.915) indicated an adequate model fit.

3.6 iELISA evaluation

The iELISA S/P cut-off calculated was 0.783 and had a test specificity Sp_{ct} of 96.9% to Cape Town sera and sensitivity Sn_{vn} of 75% to VN. There was a moderate correlation between the iELISA and VN classification (Kappa = 0.523; Supplemental Table 4) and ROC analysis had an area under the ROC curve of 0.79 (Supplemental Figure 3).

4 Discussion

Study dogs had substantial and frequent exposure to AHSV and the risk factors for exposure were consistent with midge-borne transmission. Of significance, the estimated population of AHSV-exposed dogs approximated the entire horse population in the study area. Furthermore, the frequency of exposure in dogs exceeded the reported incidence in horses in the endemic areas. Most seropositive

dogs had no clinical signs that were consistent with previous reports of AHS infection in dogs. If exposed dogs are capable of onward transmission, findings suggest that domestic dogs might have a far greater role in the AHS epidemiology in endemic areas than previously thought. Furthermore, the importation of dogs from endemic areas might pose a significant risk of AHSV introduction into AHS-disease-free areas. Study findings highlight the previously reported need for research evaluating the potential for onward AHSV transmission from exposed dogs (Oura, 2018).

The AHSV seroprevalence in dogs was similar to that reported in other endemic areas, with a weighted mean seroprevalence of 9% (Minimum = 4%; Maximum = 50%) from other studies (McIntosh, 1955; Shah, 1964; Awad et al., 1981; Baba et al., 1992; Baba et al., 1993; Alexander et al., 1995). Additionally, the seroprevalence was almost unchanged from the 7.7% reported over 60 years previously from the same study area (McIntosh, 1955). Previous studies were performed in different socioeconomic, geographic areas and years, with different testing methods. Despite these differences, the seroprevalences were similar to this study.

The study seroprevalence was low (6%) in comparison to the seroprevalence of unvaccinated donkeys in endemic areas (>50%) (Teshome et al., 2012; Gordon et al., 2017; Molini et al., 2020; Ndebé et al., 2022). Unvaccinated sentinel donkeys are used for disease surveillance as the interpretation of population serological studies in horses is complicated by high mortality and vaccination (Gordon et al., 2017). If the

current findings are representative, considering the relative population sizes, the number of seropositive dogs of 7000-10000 is roughly equal to the total number of horses in the study area. In addition study dogs seroconverted (2.3 seroconversions per 10 dog-years, 95% CI = 0.6 - 6.2) more frequently than previously reported incidence of AHS in horses (0.45 cases per 10 horse-years, 95% PI: 0.1–1.6) living in endemic areas (Sergeant et al., 2016). Should AHSV-exposed dogs be capable of onward transmission, these findings suggest that the role of dogs in AHS epidemiology could be substantial given the population size of the species.

The predominant VN antibody serotype was AHSV serotype 6 (90% of samples) and was present in all study years except 2014 (Table 1). Two VN positive dogs to serotypes 1,7,9 and 3,4 respectively did not test positive for AHSV serotype 6. Serotype 6 was responsible for dog mortalities reported from the same area (O'Dell et al., 2018) and was also reported in horses in the area during 2015/2016, along with serotypes 1,2,7,8 (Government, 2017). This serotype might represent a dog-adapted variant in terms of virulence or transmission. However, as other AHSV serotypes (1,3,4,7, and 9) have been reported to infect dogs (McIntosh, 1955; Haig, 1956; Awad et al., 1981; Alexander et al., 1995), serotype 6 might have simply been the circulating serotype at the time of the study. Antibodies to multiple serotypes were detected in four dogs and might represent cross-reactions of antibodies to different serotypes or an early non-serotype-specific humoral response. This has been reported in horses and utilised in vaccination schedules to induce immunity to cross-reactive serotypes (von Teichman et al., 2010). The median neutralization titres (1:10) observed were low but similar to vaccine responses in horses, where titres of >1:10

are considered protective (Blackburn and Swanepoel, 1988; Dennis et al., 2019; Molini et al., 2020; Rodríguez et al., 2020).

In this study, there was no apparent geographic or temporal point source for seropositivity. Two distinct non-overlapping periods when seroconversion occurred were observed implying that a single point exposure for seropositive dogs did not occur. Exposure through ingestion of AHSV-contaminated horse meat was therefore unlikely from sources such as a local butcher or a particular batch of dog food. Exposure through feeding of AHSV contaminated dead horses to farm dogs was also unlikely given the low seroprevalence observed in low-density farm areas (Figures 2 and 3).

High annual rainfall (collinear with 2017), sera collected in March, and dogs living in the middle population density quartiles (between 784-1663 and 1664-2029 people/km^2) were strongly associated with seropositivity (Table 3). A drought occurred in 2014/2015, and 2015/2016, followed by heavy rainfall in the 2016/2017 rainfall period (Figure 2). This weather pattern is associated with the warm phase of the El-Nino Southern Oscillation (ENSO) climatic phenomenon and has been linked to large AHS outbreaks in horses (Baylis et al., 1999). Heavy rainfall has been described previously in the periods preceding canine AHS mortalities (van Sittert et al., 2013; O'Dell et al., 2018). Seropositive dogs and reported AHS cases in horses had similar monthly trends with increased numbers seen in March and April (Figure 5). Findings are similar to reports of dogs in Egypt that had a higher AHSV seroprevalence in summer and spring (Awad et al., 1981). El-Nino Southern

Oscillatory-related and late summer (March, April) AHS outbreaks in horses are associated with an abundance of AHS-infected midges in South Africa (Baylis et al., 1999; Grewar et al., 2021). These risk factors associated with seropositivity support midge-borne exposure of AHSV to dogs. Study prevalence and incidence suggest that midges frequently fed on dogs increasing the species' potential for onward transmission of AHSV.

The strong association of seropositivity to the primarily suburban areas (medium density) where minimal livestock or wildlife live was surprising (Figure 4). The increased seroprevalence in suburban population density might also be explained by spillover midge-born transmission. Midges avoid canine blood meals in low-density farmland but might feed on dogs in neighboring suburban areas, due to the lack of alternative preferred hosts. Compared to suburban areas, high-density areas would be expected to have fewer watered gardens and ponds that provide the semi-moist soil needed for midge breeding sites (van Doninck et al., 2014). Additionally, most study dogs living in high-density areas were further from farmland than suburban dogs, and dogs might be less likely to be exposed to midges infected with AHSV after feeding on viraemic equids.

Only one of the iELISA seropositive dogs (n=1/22) had clinical signs potentially indicative of AHS infection at the time of blood collection. No significant associations were detected relating to diagnosis/reason for blood collection. It is therefore assumed that subclinical infection occurred in greater than 95% of the

seropositive dogs. The frequency of AHSV subclinical exposure is a further concern that subclinical dogs from endemic areas might be viraemic when imported into free areas.

The current study did not identify any breed, sex or size-related risk factors for AHSV seropositivity. Increased exposure to AHSV in Labrador retrievers was previously reported but study numbers were low limiting significance (van Sittert et al., 2013). More large breeds, short-haired and male dogs were identified in recent AHS canine mortalities (O'Dell et al., 2018) and might represent risk factors for increased AHSV virulence rather than AHSV exposure. However, as no population comparisons were reported, no firm conclusion can be made based on this previous report.

The iELISA employed in this study has not been validated for use in dogs and this is a limitation. However, the iELISA had moderate agreement and a significant AUC relative to VN suggesting its ability to be used for epidemiological investigations despite having imperfect sensitivity and specificity. iELISA S/P values overall were low and the cut-off calculated for dogs (0.783) was substantially lower than the cut-off used in equids (7.0). This might be explained by dogs having a less intense serological response to exposure or a lower binding affinity of the protein G conjugate for dog IgG antibodies compared to the affinity for equines. The AHSV cELISA assays are not species-specific (WOAH, 2022c) and might have been better suited than the iELISA for use in dogs, but they were not available in South Africa at the time of the study. The use of the iELISA as a standalone diagnostic test is not

recommended without further evaluation but can be used as a screening test with VN confirmation.

The dogs selected were concentrated around the veterinary hospital where sera were collected and are expected to be representative of the geographic distribution of dogs undergoing blood tests at the study hospital. However, there was likely a bias towards dogs owned by people able to afford veterinary care and blood testing. A potential confounding factor relating to population density and socioeconomic status might have been present but could not be investigated. Dogs used in seroconversion incidence rate calculation were biased in favour of dogs with chronic diseases (such as hypothyroidism, Cushing's, Addison's and epilepsy) requiring frequent blood testing compared to the general study population. For the calculation of population prevalence estimates, iELISA sensitivity to VN (Tshwane samples) and the iELISA specificity to Cape Town sera (AHS free) were employed. The use of these two different populations for specificity and sensitivity determination is a limitation but a single valid reference population could not be identified. The impact of these potential biases on presented results is unknown.

5 Conclusions

The current study estimated dog AHSV seroprevalence, identifying the prevailing serotypes, and risk factors associated with AHSV seropositivity in dogs in an endemic area of Tshwane. In addition to this, the incidence of AHSV exposure in dogs was also described. This study is the largest, most extensive examination of risk factors of AHSV exposure in dogs published to date. In addition, this is the first

examination of population seroprevalence in an area where AHSV mortalities in dogs have occurred. A substantial number of dogs were seropositive to AHSV and the identified risk factors were annual rainfall, human population density and the month of March. These factors are consistent with midge-borne transmission to dogs. If capable of onward transmission, study findings suggest that domestic dogs might have a far greater role in AHS epidemiology in endemic areas than previously thought. Importation of asymptomatic dogs from endemic areas might also pose a significant risk of AHSV introduction into AHS-free areas. The findings of this study highlight the need to establish if onward transmission from AHSV-infected dogs is possible.

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Tables and Figures

Table 1: A summary of the AHSV serotypes, titres and blood collection year prevalent in VN seropositive dogs (titre >1:5). The table shows the overall median and range of titres for each serotype. The overall proportion of each serotype is shown as a percentage of the total seropositive dogs (n=20) and of the total seropositive sera (n=28).

AHSV	Year of sera collection						VN Titres[+]		Seropositive	
Serotype	20	20	201	20	20	Total	Ran	Median	Sera	Dogs

	15	16	7	18	19	(sera)	ge	titre	(%)	(%)
1		1[a]				1	40	40	3.6	5.0
2		1[a]				1	10	10	3.6	5.0
3		1[a]		1[c]		2	10-20	15	7.1	10.0
4		1[a]		1[c]		2	10-30	20	7.1	10.0
5			1[b]			1	20	20	3.6	5.0
6	1	2[a]	11	2[d]	2	18	5-40	10	64.3	90.0
7			1[b]			1	10	10	3.6	5.0
8						0			0.0	0.0
9			1[b]	1[d]		2	5-40	23	7.1	10.0
Total (Dogs)	1 (1)	6 (2)	14 (12)	4 (3)	2 (2)	28 (20)	5-40	10		

AHSV: African horse sickness virus

VN Titres: Viral neutralisation titres

[+]VN titres displayed as reciprocal of dilution

[a,b,c,d] Four dogs were positive to multiple serotypes [a] (1,2,3,4,6) [b] (1,7,9) [c] (3,4) [d] (6,9)

Table 2. The univariate associations of risk factors to AHSV iELISA seropositivity of 366 dogs collected in Tshwane, South Africa between 2014 and 2019. Only variables with p <0.25 are shown (for all levels and variables evaluated see Supplemental Table 2).

Continuous variables were categorised in quartiles, apart from rainfall measures that were trichotomized evenly and where a linear trend observed evaluated as a continuous variable.

Variable	**Level**	**Number tested**	**Seropositive (%)**	**Parameter Estimate ($\hat{\beta}$)**	**Odds ratio (95% CI)**	**p-value**
Year of blood collection	2014/15 Combined	83	0 (0)	-18.13	1.00(0,.)	0.997
	2016	79	4 (5.1)	0.141	1.15 (0.30, 1.43)	0.837
	2017	91	13 (14.3)	1.281	3.60 (1.23, 10.51	0.019
	2018/19 Combined	113	5 (4.4)	Referent		0.065
	All other years	275	9 (3.2)	Referent		
	2017[+]	91	13 (14.3)	12.426	4.93 (2.03, 11.95)	<0.001
Month	*Other months	337	18 (5.3)	Referent		
	March	29	4 (13.8)	1.042	2.84 (0.89 ,9.02)	0.078

	*Other months	328	18 (5.5)	Referent		
				1.124	1.96 (0.63, 6.13)	0.246
	April	38	4 (10.5)			
Ward	*All other wards	285	14 (4.9)	Referent		
				0.752	2.12 (0.86, 5.25)	0.104
	Wards 2 and 50 Combined	81	8 (9.8)			
Ward population density	*Less than 783.7	89	2 (2.2)	Referent		0.062
				1.721	5.59 (1.13, 27.71)	0.035
	Between 783.8-1663.0	99	8 (8.0)			
	Between 1663.1 and 2029.6	93	9 (9.6)	1.794	6.01 (1.28, 28.15)	0.023
				0.684	1.98 (0.32, 12.14)	0.460
	Greater than 2029.7	85	3 (3.5)			
Annual rainfall	*Below 754mm	265	13 (3.4)	Referent		

	Greater than 754mm[+]	100	9 (13.0)	1.451	4.27(1.73, 10.33)	0.001
	Continuous			0.007	1.01(1.00, 1.01)	0.007
Sex	*Sterilised	236	17 (7.2)	Referent		
	Intact	123	4 (3.3)	-0.837	0.43 (0.14, 1.32)	0.140
	*All other	295	21 (5.3)	Referent		
	Male Intact	71	1 (1.4)	-1.65	0.19 (0.03, 1.46)	0.110
Reason for blood collection	*All other reasons	236	17 (7.2)	Referent		
	Pre-surgical blood testing	129	5 (3.9)	-0.663	0.52 (0.19, 1.43)	0.203

* Variable used in multivariable analysis.

[+] Year 2017 and high rainfall were collinear (Spearman's rho =0.93) and only rainfall measures were used for multivariate analysis

Table 3. The final variables included in a multivariable logistic regression model for the association of risk factor and AHSV iELISA seropositivity in study dogs. Only variables with univariate associations with p <0.25 were included for analysis (Table 2). Multiple logistic

regression was performed step-wise and variables with the highest Wald p-value were removed one-by-one until all variables had p-values below 0.05 (backward stepwise method).

Variable	**Level**	**Parameter estimate ($\hat{\beta}$)**	**Odds ratio (95% CI)**	**p-value**
Annual rainfall	Less than 754mm	Referent		
	Greater than 754mm	1.750	5.76 (2.22, 14.95)	<0.001
Month March	Other months	Referent		
	March	1.632	5.12 (1.41 ,18.61)	0.013
Ward population density	<= 783 people/km^2	Referent		0.032
	784 - 1663 people/km^2	1.965	7.14(1.39, 36.73)	0.019
	1664- 2029 people/km^2	1.908	6.74 (1.40, 32.56)	0.018
	2030 + people/km^2	0.618	1.86 (0.29, 11.80)	0.513

CI = confidence interval

Hosmer and Lemeshow, $\chi^2 = 2.054$, P = 0.915

Figures

Figure Captions

Figure 1. Sample selection for VN testing and test result (titres > 1:5 dilutions considered seropositive) of each selection group. For serotype detection, sera with the highest rank order iELISA S/P values (n=33) were selected for VN testing. Sera not tested from the highest iELISA S/P values (n=11) were due to inadequate sample volume (n=3), non-selection (n=5) or missing sera following iELISA testing (n=3). Additional VN tests were performed on sera with low iELISA S/P values (n=9) randomly selected from the 50th -150th rank.

Figure 2 (Separate file attached). Electoral ward map of the study location city of Tshwane, South Africa. The location of the blood bank at the Onderstepoort Veterinary Academic Hospital (White H on black) human population density (dot density), the number of dogs tested (Greyscale shading), and the number of dogs seropositive for AHSV (numbers in white boxes) overlaid per ward. Ward map boundaries courtesy of City of Tshwane, Maps and GIS, found at www.tshwane.gov.za. Population density calculated from Statistics South Africa, Census 2011. Map created using ArcGIS (Esri) software.

Figure 3. Dual-axis plot and histogram of tested, seropositive and the seroprevalence of study dogs tested using the AHSV iELISA per Tshwane electoral ward population density derived from Statistics South Africa, Census 2011.

Figure 4. Dual axis plot of quarterly seroprevalence found in study dogs using AHSV iELISA and rainfall in the study area, City of Tshwane Pretoria. The monthly and yearly rainfall is shown in the rainfall recorded and the expected rainfall (a 12-year average 2008-2020) per quarter. Rainfall data represent an average of seven weather stations across Tshwane obtained from the South African Weather Service.

Figure 5. Dual axis plot of the monthly proportion of total reported equine AHS clinical cases AHSV seropositive study dogs and average monthly rainfall (12 year average) during the study period and area City of Tshwane, South Africa. Equine data obtained from

Department of Agriculture, Land Reform and Rural Development website (found at https://dalrrd.gov.za/ disease reporting summaries). Rainfall data obtained from the South African Weather Service

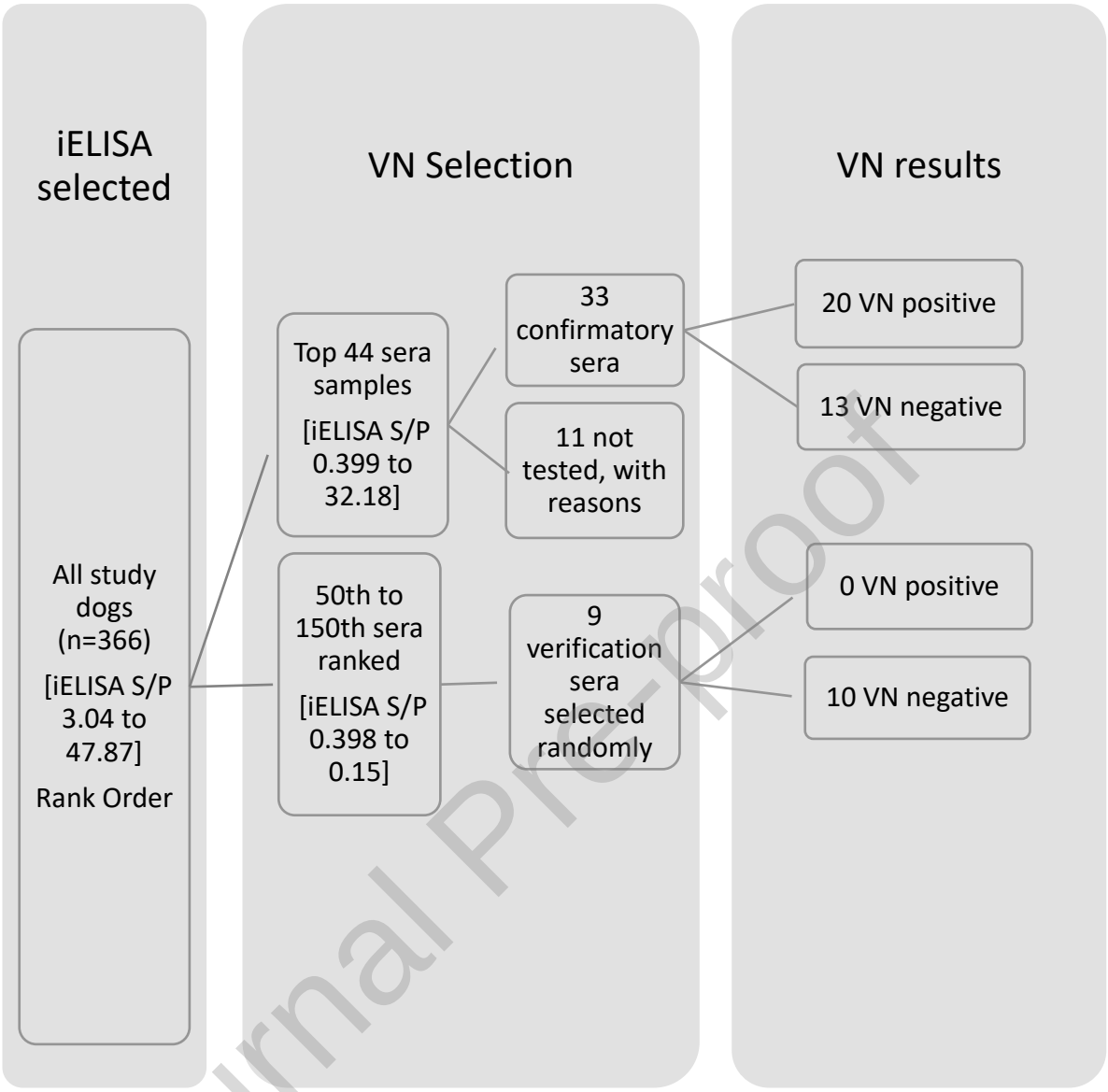


Figure 1.

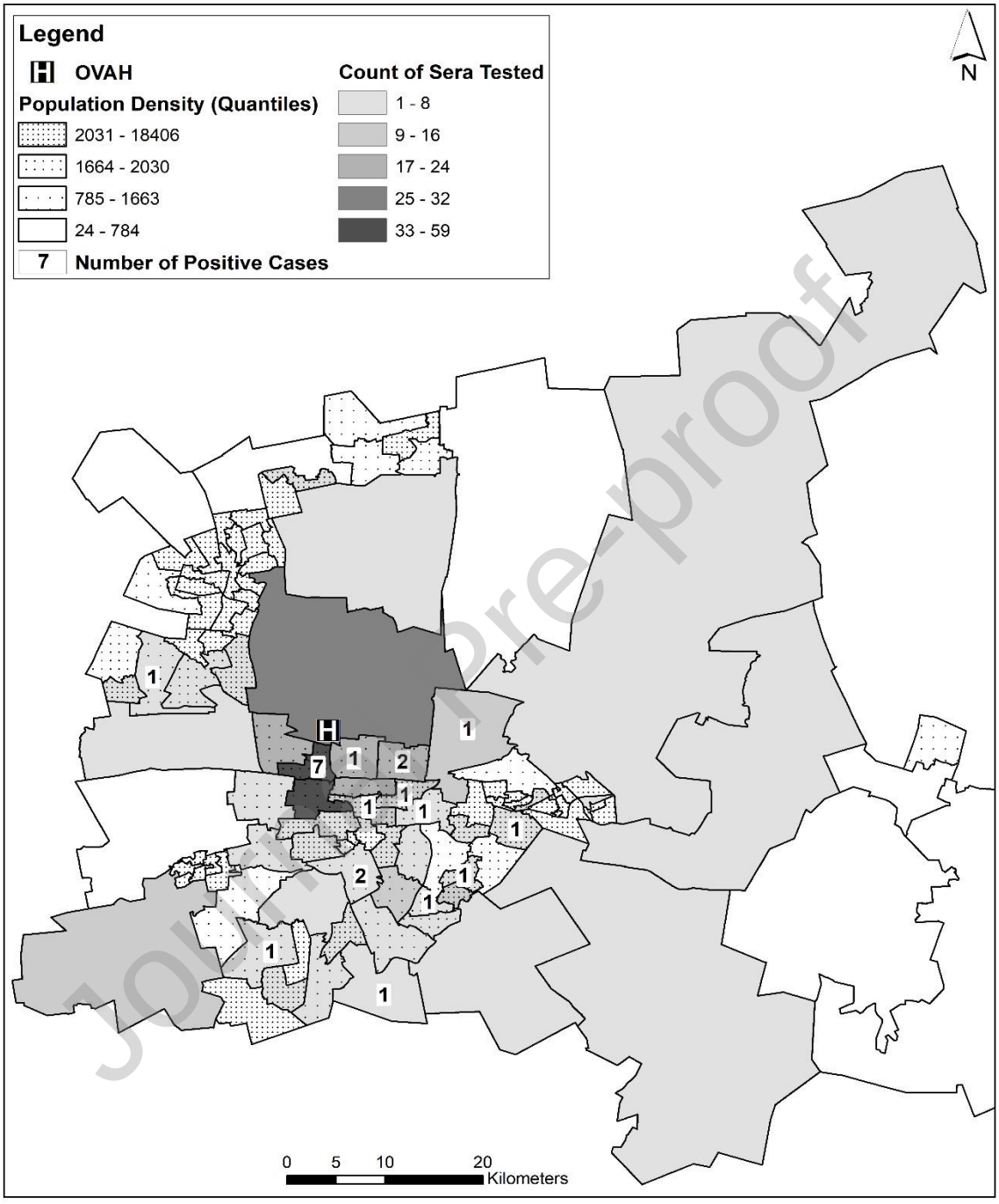
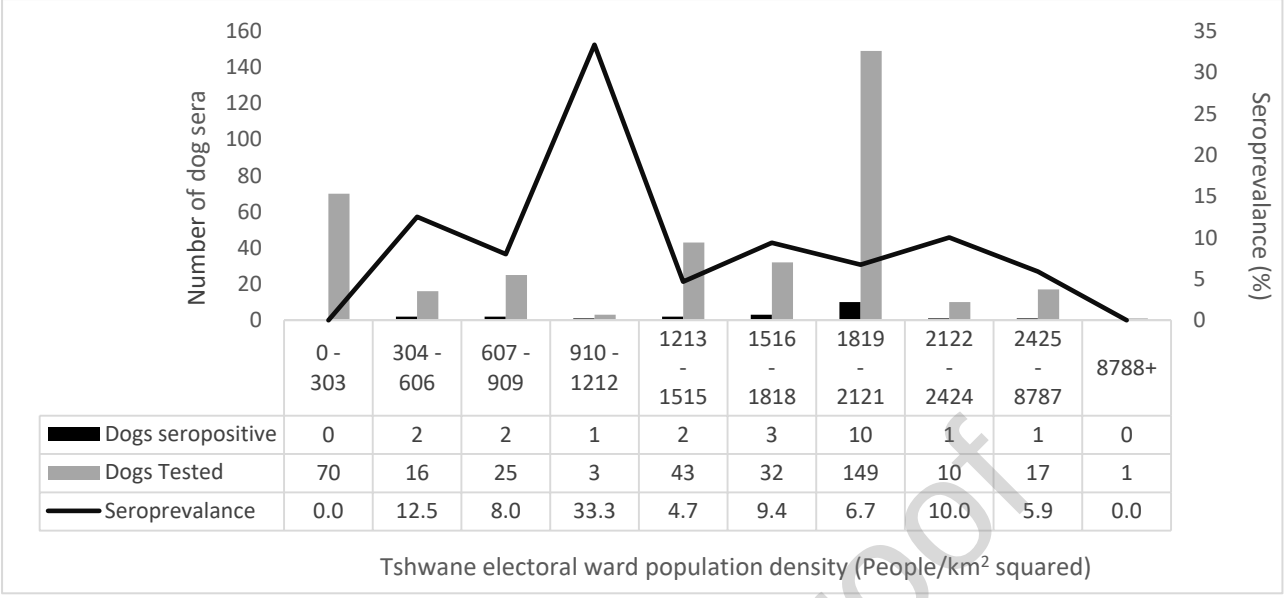

Legend
OVAH
Count of Sera Tested
Population Density (Quantiles)
1 - 8
2031 - 18406
9 - 16
1664 - 2030
17 - 24
785 - 1663
25 - 32
24 - 784
33 - 59
7 Number of Positive Cases
N
0 5 10 20
Kilometers

Figure 2.



	0 - 303	304 - 606	607 - 909	910 - 1212	1213 - 1515	1516 - 1818	1819 - 2121	2122 - 2424	2425 - 8787	8788+
Dogs seropositive	0	2	2	1	2	3	10	1	1	0
Dogs Tested	70	16	25	3	43	32	149	10	17	1
Seroprevalance	0.0	12.5	8.0	33.3	4.7	9.4	6.7	10.0	5.9	0.0

Figure 3.

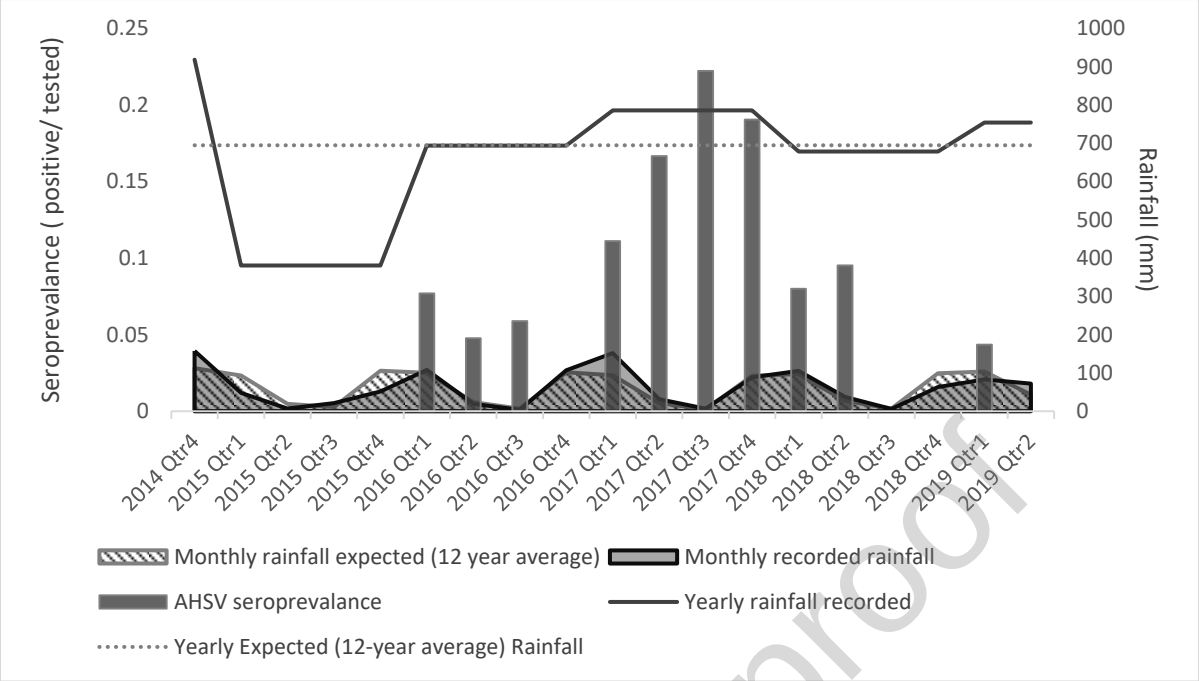


Figure 4.

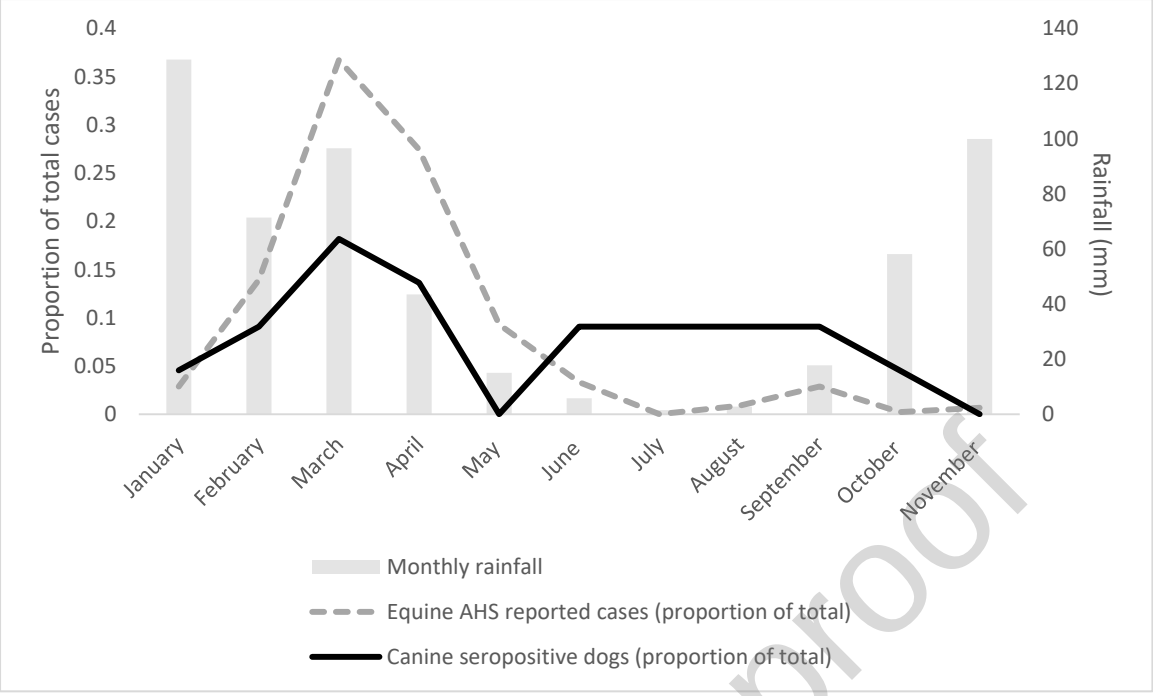


Figure 5.

Declaration of Competing Interest

☐The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Professor Geoffrey T. Fosgate serves as Co-Editor-in-Chief for the Journal. Submission is blinded and is not anticipated to influence the review process

Apendix 1: The STROBE-Vet statement checklist.

	Item	***STROBE-Vet recommendation***	***Page #***
Title and Abstract	1	(a) Indicate that the study was an observational study and, if applicable, use a common study	1 line 11

		design term	
		(b) Indicate why the study was conducted, the design, the results, the limitations, and the relevance of the findings	1
Background / rationale	2	Explain the scientific background and rationale for the investigation being reported	3
Objectives	3	(a) State specific objectives, including any primary or secondary prespecified hypotheses or their absence	5
		(b) Ensure that the level of organization[a] is clear for each objective and hypothesis	5 line 11
Study design	4	Present key elements of study design early in the paper	6 line 117
Setting	5	(a) Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6-8
		(b) If applicable, include information at each level of organization	na
Participants [b]	6	(a) Describe the eligibility criteria for the owners/managers and for the animals, at each relevant level of organization	6-7
		(b) Describe the sources and methods of selection for the owners/managers and for the animals, at each relevant level of organization	na
		(c) Describe the method of follow-up	na
		(d) For matched studies, describe matching criteria and the number of matched individuals per subject (e.g., number of controls per case)	na
Variables	7	(a) Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. If applicable, give diagnostic criteria	9
		(b) Describe the level of organization at which each variable was measured	na
		(c) For hypothesis-driven studies, the putative causal-structure among variables should be described (a diagram is strongly encouraged)	na
Data sources / measurement	8*	(a) For each variable of interest, give sources of data and details of methods of assessment (measurement). If applicable, describe comparability of assessment methods among groups and over time	9-11
		(b) If a questionnaire was used to collect data, describe its development, validation, and administration	na
		(c) Describe whether or not individuals involved in data collection were blinded, when applicable	na
		(d) Describe any efforts to assess the accuracy	na

		of the data (including methods used for “data cleaning” in primary research, or methods used for validating secondary data)	
Bias	9	Describe any efforts to address potential sources of bias due to confounding, selection, or information bias	6-7
Study size	10	(a) Describe how the study size was arrived at for each relevant level of organization	6-7
		(b) Describe how non-independence of measurements was incorporated into sample-size considerations, if applicable	na
		(c) If a formal sample-size calculation was used, describe the parameters, assumptions, and methods that were used, including a justification for the effect size selected	6-7
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen, and why	10-11
Statistical methods	12	(a) Describe all statistical methods for each objective, at a level of detail sufficient for a knowledgeable reader to replicate the methods. Include a description of the approaches to variable selection, control of confounding, and methods used to control for non-independence of observations	10-11
		(b) Describe the rationale for examining subgroups and interactions and the methods used	6-7
		(c) Explain how missing data were addressed	na
		(d) If applicable, describe the analytical approach to loss to follow-up, matching, complex sampling, and multiplicity of analyses	na
		(e) Describe any methods used to assess the robustness of the analyses (e.g., sensitivity analyses or quantitative bias assessment)	6-7
Participants	13*	(a) Report the numbers of owners/managers and animals at each stage of study and at each relevant level of organization - e.g., numbers eligible, included in the study, completing follow-up, and analyzed	6-7
		(b) Give reasons for non-participation at each stage and at each relevant level of organization	na
		(c) Consider use of a flow diagram and/or a diagram of the organizational structure	Figure 1
Descriptive data on exposures and potential	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders by group and level of	12

confounders		organization, if applicable	
		(b) Indicate number of participants with missing data for each variable of interest and at all relevant levels of organization	Figure 1
		(c) Summarize follow-up time (e.g., average and total amount), if appropriate to the study design	na
Outcome data	15*	(a) Report outcomes as appropriate for the study design and summarize at all relevant levels of organization	12-14
		(b) For proportions and rates, report the numerator and denominator	12-14
		(c) For continuous outcomes, report the number of observations and a measure of variability	12-14
Main results	16	(a) Give unadjusted estimates and, if applicable, adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders and interactions were adjusted. Report all relevant parameters that were part of the model	12-14
		(b) Report category boundaries when continuous variables were categorized	12-14
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	na
Other analyses	17	Report other analyses done,-such as sensitivity/robustness analysis and analysis of subgroups	14
Key results	18	Summarize key results with reference to study objectives	19
Strengths and Limitations	19	Discuss strengths and limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	19
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14-19
Generalizability	21	Discuss the generalizability (external validity) of the study results	14
Transparency	22	(a) Funding- Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based (b) Conflicts of interest-Describe any conflicts of interest, or lack thereof, for each author (c) Describe the authors' roles- Provision of an authors' declaration of transparency is	Title document

		recommended (d) Ethical approval- Include information on ethical approval for use of animal and human subjects (e) Quality standards-Describe any quality standards used in the conduct of the research	

[a] Level of organization recognizes that observational studies in veterinary research often deal with repeated measures (within an animal or herd) or animals that are maintained in groups (such as pens and herds); thus, the observations are not statistically independent. This non-independence has profound implications for the design, analysis, and results of these studies.
[b] The word "participant" is used in the STROBE statement. However, for the veterinary version, it is understood that "participant" should be addressed for both the animal owner/manager and for the animals themselves.
*Give such information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.